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(71) Applicant: UNIVERSITY OF PITTSBURGH [US/US]; 911 William Pitt Union, Pittsburgh, PA 15260 (US).

(72) Inventors: CHANCELLOR, Michael, B.; 5836 Ferree Street, Pittsburgh, PA 15217 (US). HUARD, Johnny; 6412 Howe Street, Pittsburgh, PA 15206 (US).

(74) Agents: SERUNIAN, Leslie, A. et al.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US).

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(57) Abstract

The present invention provides muscle-derived cells, preferably myoblasts and muscle-derived stem cells, genetically engineered to contain and express one or more heterologous genes or functional segments of such genes, for delivery of the encoded gene products at or near sites of musculoskeletal, bone, ligament, meniscus, cartilage or genitourinary disease, injury, defect, or dysfunction. *Ex vivo* myoblast mediated gene delivery of human inducible nitric oxide synthase, and the resulting production of nitric oxide at and around the site of injury, are particularly provided by the invention as a treatment for lower genitourinary tract dysfunctions. *Ex vivo* gene transfer for the musculoskeletal system includes genes encoding acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor, platelet derived growth factor, transforming growth factor-β, transforming growth factor-α, nerve growth factor and interleukin-1 receptor antagonist protein (IRAP), bone morphogenetic protein (BMPs), cartilage derived morphogenetic protein (CDMPs), vascular endothelial growth factor (VEGF), and sonic hedgehog proteins.

MUSCLE-DERIVED CELL MEDIATED GENE DELIVERY FOR TREATING MUSCLE- AND BONE-RELATED INJURY OR DYSFUNCTION

FIELD OF THE INVENTION

The present invention relates generally to compositions and methods comprising myogenic or muscle-derived cells, including myoblasts and muscle-derived stem cells for tissue engineering and cell-mediated gene therapy. The invention further relates to the introduction of exogenous nucleic acids into muscle-derived cells, including myoblasts and muscle-derived stem cells, resulting in the expression of one or more gene products by the genetically engineered muscle-derived cells. Such engineered cells are then capable of producing the gene products and effecting an enhanced physiological response after administration to a recipient host, including humans.

BACKGROUND OF THE INVENTION

A number of defects, diseases and pathological conditions in a variety of areas of medicine would benefit from the development of noninvasive treatments utilizing improved gene delivery vehicles and systems that allow the safe, efficient and sustained production of gene products to an affected tissue or organ site. In particular, improved cell-mediated gene delivery vehicles and methods would find wide use in ameliorating non-fatal, yet debilitating, pathologies of the musculoskeletal system, such as arthritis and joint disease (e.g., ligament, meniscus and cartilage); the bone, such as segmental bone defects and non-unions; and the genitourinary system, such as urinary incontinence and bladder conditions.

Although synovial cells have been used to deliver potentially therapeutic agents into the joint, the expression of such agents has declined over time, thereby causing these agents generally to become

undetectable after about four to six weeks. (G. Bandara et al., 1993, *Proc. Natl. Acad. Sci. USA*, 90(22):10764-10768; C.H. Evans and P.D. Robbins, 1995, *Ann. Med.*, 27(5):543-546; C.H. Evans and P.D. Robbins, 1994, *J. Rheum.*, 21(5):779-782). This decline in expression over time may be ameliorated by the use of cell mediated gene delivery employing a myogenic cell type that becomes post-mitotic with differentiation, in accordance with the present invention.

Segmental bone defects and non-unions are relatively common problems facing all orthopedic surgeons. Osteogenic proteins, e.g., bone morphogenic protein-2, BMP-2), can promote bone healing in segmental bone defects. However, a large quantity of the human recombinant protein is needed to enhance bone healing potential. Moreover, current modes of delivering such quantities of protein, i.e., a biological allograft or a synthetic carrier, are hampered by limited availability, possible disease transmission and the need for further research and investigation.

Cell mediated gene therapy in the bone defect would allow a sustained expression of osteogenic proteins, further enhance bone healing, and offer a solution to the problems surrounding current methods of bone protein delivery. Thus, in accordance with the present invention, the utilization of muscle-derived cells, e.g., myoblasts, as cellular gene delivery vehicles to correct or improve a bone defect, provides an important step in establishing a less invasive treatment for non-unions and segmental bone defects.

Ex vivo gene therapy and myoblast transplantation are two closely related methods which require *in vitro* cell isolation and culture. *Ex vivo* techniques involve muscle biopsy and myogenic cell isolation (T.A. Rando et al., 1994, *J. Cell Biol.*, 125:1275-1287; Z. Qu et al., 1998, *J. Cell Biol.*, 142(5):1257-1267). The isolated muscle-derived cells are transduced *in vitro* with the desired gene carrying vector. The satellite cells are then

reinjected into skeletal muscle, fuse to form post-mitotic myotubes and myofibers, and begin growth factor production. This technique is feasible with adenoviral, retroviral, and herpes simplex viral vectors.

The following are examples of orthopaedic applications for muscle based gene therapy and tissue engineering related to the practice of the present invention:

Muscle Injury and Repair

Muscle injuries comprise a large percentage of recreational and competitive athletic injuries. Muscle injuries may result from both direct (e.g., contusions, lacerations) and indirect (e.g., strains, ischemia and neurological injuries) trauma. Upon injury, satellite cells are released and activated in order to differentiate into myotubes and myofibers, thereby promoting muscle healing. However, this reparative process is usually incomplete and accompanied by a fibrous reaction producing scar tissue. This scar tissue limits the muscle's potential for functional recovery (T. Hurme et al., 1991; *Med. Sci. Sports Exerc.*, 23:801-810; T. Hurme et al., 1992, *Med. Sci. Sports Exerc.*, 24:197-205).

Investigations in animals have identified possible clinical applications for muscle-based tissue engineering to treat muscle injuries (W.E. Garrett et al., 1984; *J. Hand Surgery (Am).* 9A:683-692; W.E. Garrett et al., 1990, *Med. Sci. Sports Exerc.*, 22:436-443). Injured skeletal muscle releases numerous growth factors acting in autocrine and paracrine fashion to modulate muscle healing. These proteins activate satellite cells to proliferate and differentiate into myofibers (T. Hurme, 1992, *Med. Sci. Sports Exerc.*, 24:197-205; R. Bischoff, 1994, "The satellite cell and muscle regeneration". *Myology.* 2nd Edition. New York, McGraw-Hill, Inc, pp.97-118; H.S. Allamedine et al., 1989; *Muscle Nerve*, 12:544-555; E. Schultz et al., 1985, *Muscle Nerve*, 8:217; E. Schultz, 1989, *Med. Sci. Sports Exerc.*, 21:181).

Muscle-based tissue engineering offers exciting potential

therapies for muscle disorders. A large number of recreational and professional athletic injuries involve skeletal muscle (Garrett et al., 1990, *Med. Sci. Sports Exerc.*, 22:436-443). Therapies to improve functional recovery and shorten rehabilitation may both optimize performance and minimize morbidity. Further research is ongoing to refine these muscle-based tissue engineering applications. The results of such investigations may provide revolutionary treatments for these common muscle injuries. The present invention provides new and exciting treatments for muscle repair following muscle-based injuries, particularly for application in clinical settings.

Bone Healing

Multiple surgical specialties, including orthopaedic, plastic, and maxillofacial, are concerned with bone healing augmentation. Physicians in these disciplines rely on bone augmentation techniques to improve healing of fracture non-unions, oncologic and traumatic bone defect reconstructions, joint and spine fusions, and artificial implant stabilizations. Unfortunately, current techniques of autograft, allograft, and electrical stimulation are often suboptimal. Therefore, tissue engineering approaches toward bone formation have immense implications.

Intramuscular bone formation is a poorly understood phenomenon. It can be present in the clinically pathologic states of heterotopic ossification, myositis ossificans, fibrodysplasia ossificans progressiva and osteosarcoma. Radiation therapy and the anti-inflammatory drug, indomethicin, can suppress myositis ossificans. However, neither the mechanism of formation nor suppression of ectopic bone is clearly understood. A growing family of bone morphogenetic proteins (BMPs), members of the transforming growth factor β (TGF-β) superfamily, are recognized as being capable of stimulating intramuscular bone. Human BMP-2 in recombinant form (rhBMP-2) and BMP-encoding cDNA contained in a plasmid construct induce bone formation when

injected into skeletal muscle (E.A. Wang et al., 1990, *Proc. Natl. Acad. Sci. USA*, 87:2220-2224; J. Fang et al, 1996, *Proc. Natl. Acad. Sci. USA*, 93:5753-5758). Current applications focus on injecting rhBMP-2 directly into non-unions and bone defects. However, muscle-based tissue engineering has enormous promise in the arena of bone healing and may shed light on the physiologic mechanism of ectopic bone formation.

Intraarticular Disorders

Degenerative and traumatic joint disorders are encountered frequently as our population becomes more active and lives longer. These disorders include arthritis of various etiologies, ligament disruptions, meniscal tears, and osteochondral injuries. Currently, the clinician's tools consist primarily of surgical procedures aimed at biomechanically altering the joint, such as anterior cruciate ligament (ACL) reconstructions, total knee replacement, meniscal repair or excision, cartilage debridement, etc.. Tissue engineering applied to these intraarticular disease states theoretically offers a more biologic and less disruptive reparative process.

Both direct (I. Nita et al., 1996, *Arthritis Rheum.*, 39:820-828) and *ex vivo* (G. Bandara et al., 1993, *Proc. Natl. Acad. Sci. USA*, 90:10764-10768) gene therapy approaches to arthritis models have been reported. The synovial cell-mediated *ex vivo* approach, while offering advantages of *ex vivo* gene transfer such as the safety of *in vitro* genetic manipulation and precise cell selection, is hindered by a decline of gene expression after 5-6 weeks (Bandara et al., 1993, *Ibid.*). Due to its ability to form post-mitotic myotubes and myofibers, the satellite cell offers the theoretical advantages of longer term and more abundant protein production.

Muscle cell-mediated *ex vivo* gene delivery to numerous intraarticular structures is possible. Intraarticular injection of primary myoblasts, transduced by adenovirus carrying the β-galactosidase marker gene, results in gene delivery to many intraarticular structures (C.S. Day et al., 1997, *J. Orthop. Res.*, 15:227-234). Tissues expressing β-

galactosidase at 5 days after injection in the rabbit knee include the synovial lining, meniscal surface, and cruciate ligament (*Ibid.*). In contrast, injection of transduced synovial cells results in β-galactosidase expression only in the synovium (*Ibid.*). Likewise, injection of transduced immortalized myoblasts results in gene delivery to various intraarticular structures, including the synovial lining and patellar ligament surface. However, the purified immortalized myoblasts fused more readily and resulted in more *de novo* intraarticular myofibers than the primary myoblasts. This illustrates the importance of obtaining a pure population of myogenic cells, void of the fibroblast and adipocyte contamination often seen in primary myoblasts.

Muscle cell-mediated *ex vivo* approaches are predicated on myoblast fusion to form myofibers, the plurinuclear protein-producing factories. Intraarticular injection of transduced immortalized myoblasts into a severe combined immune deficient (SCID) mouse results in myotube formation and transgene expression in multiple structures at 35 days. Therefore, intraarticular gene expression (for at least 35 days) resulting from muscle cell-mediated tissue engineering is feasible in animal models.. Based on this data, a muscle cell-mediated gene transfer approach may deliver genes to improve the healing of several intraarticular structures specifically to the ACL and meniscus.

The ACL is the second most frequently injured knee ligament. Unfortunately, the ACL has a low healing capacity, possibly secondary to its encompassing synovial sheath or the surrounding synovial fluid. Because complete tears of the ACL are incapable of spontaneous healing, current treatment options are limited to surgical reconstruction using autograft or allograft. The replacement graft, often either patella ligament or hamstring tendon in origin, undergoes ligamentization with eventual collagen remodeling (S.P. Arnosczky et al., 1982, *Am. J. Sports Med.*, 10:90-95). Therefore, augmentation of this ligamentization process using growth factors to affect fibroblast behavior is envisioned by the practice of

the methods described herein. *In vivo* data suggests that platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and epidermal growth factor (EGF) promote ligament healing (N.A. Conti et al., 1993, *Trans Orthop. Res. Soc.*, 18:60). Transient, low levels of these growth factors resulting from their direct injection into the injured ligament are unlikely to produce a significant response. Therefore, an efficient delivery mechanism is essential to the development of a clinically applicable therapy. Muscle cell-mediated *ex vivo* gene therapy according to the teachings herein offers the potential to achieve persistent local gene expression and subsequent growth factor delivery to the ACL.

With more specific regard to the knee, the knee meniscus plays a critical role in maintaining normal knee biomechanics. Primary functions of the meniscus include load transmission, shock absorption, joint lubrication, and tibiofemoral stabilization in the ACL deficient knee. The historical treatment of menisectomy for meniscal tears has been replaced by meniscal repair when tears involve the meniscus' peripheral, vascular third. Growth factors, including platelet-derived growth factor (PDGF), are capable of enhancing meniscal healing (K.P. Spindler et al., 1995, *J. Orthop. Res.*, 13(2):201-207). However, needed for both the practitioner and the patient are better methods and procedures to deliver such needed factors to the meniscus to provide healing and repair.

Urologic Applications

Urinary incontinence is a devastating medical and social condition. The incidence of urinary incontinence is increasing in the United States due to an aging population. As of January 1997, the National Institute of Diabetes and Digestive and Kidney Disease has launched a public health campaign to address the fact that there are over eleven million women and four million men in the United States who have urinary incontinence problems. Approximately half of the fifteen million people with incontinence have stress urinary incontinence; however, less than half of

the afflicted people are seeking help and receiving the treatments which are available (Agency for Health Care Policy and Research, AHCPR, 1992 and 1996).

Presently, the estimated annual cost for treating people with urinary incontinence is over $16 billion in the United States. Most of this money is spent on management measures, such as adult diapers and pads, rather than on treatment. Since most of the invasive and surgical treatment for urinary incontinence involves the treatment of stress urinary incontinence, the cost for managing stress urinary incontinence is estimated at $9 billion dollars per year in the United States (AHCPR 1996).

In evaluating an individual with incontinence, three of the most common types and causes of incontinence can be identified: a) urge incontinence, b) stress incontinence, or c) overflow incontinence (M.B. Chancellor and J.G. Blaivas, 1996, *Atlas of Urodynamics*, Williams and Wilkins, Philadelphia, PA.).

Stress incontinence is the involuntary loss of urine during coughing, sneezing, laughing, or other physical activities which increase abdominal pressure. This condition may be confirmed by observing urine loss coincident with an increase in abdominal pressure, in the absence of a bladder contraction or an overdistended bladder. The condition of stress incontinence may be classified as either urethral hypermobility or intrinsic sphincter deficiency. In urethral hypermobility, the bladder neck and urethra descend during cough or strain on urodynamic and the urethra opens with visible urinary leakage (leak point pressure between 60-120 cm H_20). In intrinsic sphincter deficiency, the bladder neck opens during bladder filling without bladder contraction. Visible urinary leakage is seen with minimal or no stress. There is variable bladder neck and urethral descent, often none at all, and the leak point pressure is low (< 60 cm H_20). (J.G. Blaivas, 1985, *Urol. Clin. N. Amer.*, 12:215-224; D.R. Staskin et al., 1985, *Urol. Clin. N. Amer.*, 12:271-278).

Urge incontinence is defined as the involuntary loss of urine associated with an abrupt and strong desire to void. Although involuntary bladder contractions can be associated with neurologic disorders, they can also occur in individuals who appear to be neurologically normal (P. Abrams et al., 1987, *Neurol. & Urodynam.*, 7:403-427). Common neurologic disorders associated with urge incontinence are stroke, diabetes, and multiple sclerosis (E.J. McGuire et al, 1981, *J. Urol.*, 126:205-209). Urge incontinence is caused by involuntary detrusor contractions that can also be due to bladder inflammation and impaired detrusor contractility where the bladder does not empty completely.

Overflow incontinence is characterized by the loss of urine associated with overdistension of the bladder. Overflow incontinence may be due to impaired bladder contractility or to bladder outlet obstruction leading to overdistension and overflow. The bladder may be underactive secondarily to neurologic conditions such as diabetes or spinal cord injury, or following radical pelvic surgery.

Another common and serious cause of urinary incontinence (urge and overflow type) is impaired bladder contractility. This is an increasingly common condition in the geriatric population and in patients with neurological diseases, especially diabetes mellitus (N.M. Resnick et al., 1989, *New Engl. J. Med.*, 320:1-7; M.B. Chancellor and J.G. Blaivas, 1996, *Atlas of Urodynamics*, Williams and Wilkins, Philadelphia, PA). With inadequate contractility, the bladder cannot empty its content of urine; this causes not only incontinence, but also urinary tract infection and renal insufficiency. Presently, clinicians are very limited in their ability to treat impaired detrusor contractility. There are no effective medications to improve detrusor contractility. Although urecholine can slightly increase intravesical pressure, it has not been shown in controlled studies to aid effective bladder emptying (A. Wein et al., 1980, *J. Urol.*, 123:302). The most common treatment is to circumvent the problem with intermittent or

indwelling catheterization.

There are a number of treatment modalities for stress urinary incontinence. The most commonly practiced current treatments for stress incontinence include the following: absorbent products; indwelling catheterization; pessary, i.e., vaginal ring placed to support the bladder neck; and medication (Agency for Health Care Policy and Research. Public Health Service: Urinary Incontinence Guideline Panel. Urinary Incontinence in Adults: Clinical Practice Guideline. AHCPR Pub. No. 92-0038. Rockville, MD. U.S. Department of Health and Human Services, March 1992; M.B. Chancellor, Evaluation and Outcome. In: The Health of Women With Physical Disabilities: Setting a Research Agenda for the 90's. Eds. Krotoski D.M., Nosek, M., Turk, M., Brooks Publishing Company, Baltimore, MD, Chapter 24, 309-332, 1996). With specific regard to medication, there are several drugs approved for the treatment of urge incontinence. However, there are no drugs approved or effective for stress urinary incontinence.

Exercise is another treatment modality for stress urinary incontinence. For example, Kegel exercise is a common and popular method to treat stress incontinence. The exercise can help half of the people who can do it four times daily for 3-6 months. Although 50% of patients report some improvement with Kegel exercise, the cure rate for incontinence following Kegel exercise is only 5 percent. In addition, most patients stop the exercise and drop out from the protocol because of the very long time and daily discipline required.

Another treatment method for urinary incontinence is the urethral plug. This is a new, inexpensive disposable cork-like plug for women with stress incontinence. A new plug should be used after each micturition, with an estimated daily cost of about $15-20. The estimated annual disposable cost is over $5,000. The plug is associated with over 20% urinary tract infection and, unfortunately, does not cure incontinence.

Biofeedback and functional electrical stimulation using a

vaginal probe are also used to treat urge and stress urinary incontinence. However, these methods are time-consuming and expensive and the results are only moderately better than Kegel exercise. Surgeries, such as laparoscopic or open abdominal bladder neck suspensions; transvaginal approach abdominal bladder neck suspensions; artificial urinary sphincter (expensive complex surgical procedure with 40% reversion rate) are also used to treat stress urinary incontinence.

Other treatments include urethra injection procedures with exogenous injectable materials such as Teflon, collagen, and autologous fat. Each of these injectables has its disadvantages. More specifically, there are significant reservations among those in the medical community concerning the use of Teflon. Complications of Teflon injection include granuloma, diverticulum, cysts, and urethral polyp formation. Of greatest concern is the migration (via the lymphatic and vascular systems) of Teflon particles to distant locations, resulting in fever and pneumonitis.

Collagen injections generally employ bovine collagen, which is expensive and is often reabsorbed, resulting in the need for repeated injections. A further disadvantage of collagen is that about 5% of patients are allergic to bovine source collagen and develop antibodies.

Autologous fat grafting as an injectable bulking agent has a significant drawback in that most of the injected fat is resorbed. In addition, the extent and duration of the survival of an autologous fat graft remains controversial. An inflammatory reaction generally occurs at the site of implant. Complications from fat grafting include fat resorption, nodules and tissue asymmetry.

In view of the above-mentioned limitations and complications of treating urinary incontinence and bladder contractility, new and effective modalities in this area are needed in the art. In accordance with the present invention, muscle cell injection therapy using uniquely engineered muscle-derived cells is provided as an improved and novel means for

treating and curing various types of incontinence, particularly, stress urinary incontinence and for the enhancement of urinary continence. As but one advantage, muscle-derived cell injection can preferably be autologous, so that there will minimal or no allergic reactions, unlike the aforementioned use of collagen. Also, unlike collagen, myogenic cells such as blasts are not absorbed; thus, they can provide a better improvement and cure rate.

Myoblasts, the precursors of muscle fibers, are mononucleated muscle cells which differ in many ways from other types of cells. Myoblasts naturally fuse to form post-mitotic multinucleated myotubes which result in the long-term expression and delivery of bioactive proteins (T.A. Partridge and K.E. Davies, 1995, *Brit. Med. Bulletin*, 51:123-137; J. Dhawan et al., 1992, *Science*, 254: 1509-1512; A.D. Grinnell, 1994, In: *Myology*. Ed 2, Ed. Engel AG and Armstrong CF, McGraw-Hill, Inc, 303-304; S. Jiao and J.A. Wolff, 1992, *Brain Research*, 575:143-147; H. Vandenburgh, 1996, *Human Gene Therapy*, 7:2195-2200). Myoblasts have been used for gene delivery to muscle for muscle-related diseases, such as Duchenne muscular dystrophy (E. Gussoni et al., 1992, *Nature*, 356:435-438; J. Huard et al., 1992, *Muscle & Nerve*, 15:550-560; G. Karpati et al., 1993, *Ann. Neurol.*, 34:8-17; J.P. Tremblay et al., 1993, *Cell Transplantation*, 2:99-112), as well as for non-muscle-related diseases, e.g., gene delivery of human adenosine deaminase for the adenosine deaminase deficiency syndrome (C.M. Lynch et al., 1992, *Proc. Natl. Acad. Sci. USA*, 89:1138-1142); gene transfer of human proinsulin for diabetes mellitus (G.D. Simonson et al., 1996, *Human Gene Therapy*, 7:71-78); gene transfer for expression of tyrosine hydroxylase for Parkinson's disease (S. Jiao et al., 1993, *Nature*, 362:450); transfer and expression of Factor IX for hemophilia B (Y. Dai et al., 1995, *Proc. Natl. Acad. Sci. USA*, 89:10892), delivery of human growth hormone for growth retardation (J. Dhawan et al., 1992, *Science*, 254:1509-1512).

The use of myoblasts to treat muscle degeneration, to repair

tissue damage or treat disease is disclosed in U.S. Patent Nos. 5,130,141 and 5,538,722. Also, myoblast transplantation has been employed for the repair of myocardial dysfunction (S.W. Robinson et al., 1995, *Cell Transplantation*, 5:77-91; C.E. Murry et al., 1996, *J. Clin. Invest.*, 98:2512-2523; S. Gojo et al., 1996, *Cell Transplantation*, 5:581-584; A. Zibaitis et al., 1994, *Transplantation Proceedings*, 26:3294).

Nitric oxide (NO) has been recognized as a important transmitter in genitourinary tract function. NO mediates smooth muscle relaxation and is also the key to achieving erection. Recently, constitutive and inducible nitric oxide synthase (NOS or iNOS) have been demonstrated in the urothelium, bladder and urethra wall. A deficiency in urinary NO in patients having interstitial cystitis bladder inflammation (M.A. Wheeler et al., 1997, *J. Urol.*, 158(6):2045-2050; S.D. Smith et al., 1997, *J. Urol.*, 158(3 Pt 1):703-708). Moreover, patients with interstitial cystitis had improvement in urinary symptoms and increased urinary NO production when treated with oral L-Arginine (M.A. Wheeler et al., 1997, *J. Urol.*, 158(6):2045-2050). Recent evidence has shown that urethral smooth muscle relaxation is mediated by NO release and that NO also mediates prostate smooth muscle relaxation (H. Kakizaki et al., 1997, *Am. J. Phys.*, 272:R1647-1656; A.L. Burnett, 1995, *Urology*, 45:1071-1083; M. Takeda et al., 1995, *Urology*, 45:440-446; W. Bloch et al., 1997, *Prostate*, 33:1-8).

SUMMARY OF THE INVENTION

It is an object of the present invention to provide new and effective methods and compositions for the treatment of various types of disease conditions and defects of the musculoskeletal system and the bone, using genetically engineered muscle-derived cells, e.g., myoblasts and muscle-derived stem cells, in the cell-mediated delivery of exogenous genes for the expression and production of encoded gene products. The present invention affords a stable gene delivery vehicle to afflicted areas, e.g., the joint (ligament, meniscus, and cartilage), smooth muscle, skeletal

muscle and bone, which sustains the production of proteins that ameliorate pathological muscle-related conditions, e.g., musculoskeletal and bone conditions. Examples of pathological conditions of the musculoskeletal system include arthritis and damage to ligaments, cartilage and meniscus, resulting in general muscle weakness and/or dysfunction, such as in the face and hands, as a nonlimiting example. Disease conditions of the bone include segmental bone fractures, defects, weakness, non-unions and any type of bone augmentation. The present invention overcomes the problem of transient gene expression, which reduces the efficacy of *ex vivo* gene transfer to the joint, for example, using synovial cells.

Another object of the present invention is to provide a general method for muscle-derived cell mediated *ex vivo* gene transfer involving harvesting muscle-derived cells, preferably, autologous muscle-derived cells, culturing the cells, transducing the cultured cells with an appropriate vector *in vitro*, e.g., a viral vector, harboring at least one exogenous gene encoding a bioactive molecule, such as a protein, polypeptide, peptide, drug, enzyme, metabolite, hormone and the like, and injecting the transduced muscle-derived cells into or near an affected area or site of injury, for example, a muscle; a joint, preferably, the knee joint; a bone defect; or a genitourinary tract defect.

According to the present invention, the method further includes enhancing and/or ameliorating the therapeutic and repair effects of the expressed bioactive molecule using muscle-derived cell mediated gene transfer to co-deliver gene(s) coding for trophic factors, e.g., growth factors, or auxiliary proteins and the like, which are also functionally expressed to further promote and ameliorate treatment and repair of the affected tissue. Suitable muscle-derived cells for use are myoblasts.

Also in accordance with the invention, the candidate molecules to be delivered with the muscle based gene therapy and tissue engineering include bone morphogenetic protein (BMP)-2, (BMP-2), and

other subtypes of BMP (e.g. BMP-6 and BMP-12), vascular endothelial growth factor (VEGF), cartilage-derived morphogenetic proteins 1, 2 (CDMP-1, 2) and hedgehog, for the improvement of bone and cartilage healing. Insulin like growth factor- 1 (IGF-1), nerve growth factor and basic fibroblast growth factor (bFGF) are used to improve muscle healing following injuries. In fact, according to the present invention, the use of these recombinant human growth factor proteins has been shown to improve muscle healing following laceration, contusion and strain injuries.

To improve the healing of meniscal injuries, epidermal growth factor (EGF), transforming growth factor α (TGF- α), basic fibroblast growth factor (bFGF) and platelet derived growth factor A,B (PDGF-A,B) are useful, since these growth factors are capable of improving meniscal fibrochondrocyte proliferation and increasing the synthesis of collagen and non-collagen proteins.

Finally, the growth factors particularly suited to improve ligament healing include platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), and epidermal growth factor (EGF), which are capable of improving the proliferative capacity of ligament fibroblast and therefore are important candidates to improve ligament healing.

It is another object of the present invention to provide new and effective methods and compositions for the treatment of various types of urinary incontinence, particularly urinary stress incontinence, using genetically engineered muscle-derived cells in the cell-mediated delivery of exogenous genes and their encoded gene products to tissues of the urinary system, such as the urethra and bladder.

It is yet another object of the present invention to provide uniquely engineered muscle-derived cells for carrying genes encoding products for treating a number of genetic and pathologic conditions of the musculoskeletal system and for treating and curing various types of

incontinence, as well as for the further enhancement of urinary continence. Suitable muscle-derived cells include myoblasts and muscle-derived stem cells that will eventually differentiate into myotubes and muscle fibers, as well as into other lineages such as osteoblasts, chondrocytes, and smooth muscle cells, in particular, when muscle-derived stem cells are used.

Another object of the present invention to inject autologous muscle-derived cells (e.g., myoblasts, and muscle-derived stem cells) that have been transfected or transduced with a vector (e.g., viral and non-viral) containing at least one gene encoding a bioactive molecule and, optionally, at least one gene encoding a trophic factor, e.g., a growth factor or a neurotropic factor, into a muscle tissue, e.g., the urethral wall as an effective treatment for stress urinary incontinence. The muscle-derived cells can be cultured and harvested and can generate sufficient quantities of muscle cells for repeated injections. The present invention is intended to embrace muscle-derived cells which have been genetically engineered to contain genes encoding both a bioactive molecule and a trophic factor. Alternatively, different muscle-derived cells can be engineered to contain either a gene encoding a bioactive molecule or a gene encoding a trophic factor or an immune suppression agent. The different muscle-derived cells can be co-injected or injected at different times, or in combination with other transduced muscle-derived cells, depending upon the type of treatment and therapeutic enhancement desired.

In accordance with the present invention, muscle-derived cells expressing desired gene products comprise a beneficial cell-mediated gene therapy which allows the survival of injected cells and the persistence of gene products, including growth factors (e.g. bFGF, IGF-1, VEGF, PDGF A,B, BMP-2, CDMP, etc.) and neurotropic factors (e.g., nerve growth factor) to treat and improve urinary tract dysfunction over prolonged periods of time.

Yet another object of the present invention is to provide a

simple treatment method for women and men with stress urinary incontinence by using autologous, transfected muscle-derived cells to enhance their urinary sphincters. Such muscle-derived cell-mediated gene therapy allows repair and improvement of the urinary sphincter. In accordance with the present invention the treatment comprises a simple needle aspiration to obtain muscle-derived cells, for example, and a brief follow-up treatment to inject cultured and prepared cells into the patient via an outpatient endoscopic procedure. Also according to the present invention, autologous muscle cell injections using myoblasts and muscle-derived stem cells harvested from and cultured for a specific stress incontinence patient can be employed as a nonallergenic agent to bulk up the urethral wall, thereby enhancing coaptation and improving the urinary sphincter muscle. In this aspect of the invention, simple autologous muscle cell transplantation is performed, preferably without an accompanying gene therapy.

Another object of the present invention is the use of genetically engineered muscle-derived cells and cell mediated gene delivery for injection into the detrusor muscle as a means of modulating bladder contractility. In accordance with the present invention, survival of the muscle-derived cells and the expression of foreign genes in such cells have been demonstrated after injection into the bladder wall. Muscle-derived cell mediated gene therapy provides a useful treatment for modulating detrusor contractility and for an overactive bladder.

Another object of the present invention is to provide genetically engineered muscle-derived cells (e.g., myoblasts and muscle-derived stem cells) carrying the nitric oxide synthase (NOS) gene, preferably, inducible NOS (iNOS), for expression of nitric oxide synthase as a therapy for genitourinary tract dysfunction, for example, male erectile dysfunction, bladder inflammation, or stress incontinence. In accordance with the present invention, muscle-derived cells carrying the iNOS gene

have been demonstrated to successfully deliver the inducible form of NOS (iNOS) into the penis and genitourinary tissue. Moreover, the production of iNOS, which produces higher quantities of nitric oxide than is produced by constitutive NOS, by the genetically engineered muscle-derived cells stimulated the release of NO and provided a significant increase in intracavernosal pressure, which was mediated by NO-induced penile vasodilation.

Further objects and advantages afforded by the present invention will be apparent from the detailed description and exemplification hereinbelow.

DESCRIPTION OF THE DRAWINGS

The appended drawings of the figures are presented to further describe the invention and to assist in its understanding through clarification of its various aspects.

Figs. 1A-1I present light and fluorescence microscopic analyses of urethral and neck tissue demonstrating the persistence of injected myoblasts carrying the ß-galactosidase gene and producing ß-galactosidase, i.e., lacZ, (blue spots) and fluorescent latex microspheres (fluorescent green). Increasing magnification (from 40x to 100x) of the same specimen is shown, with Figs. 1A, 1D, and 1G having the lowest magnification and Figs. 1C, 1F, and 1I having the highest magnification. **Figs. 1A-1C** represent bladder neck myoblast injection. **Figs. 1D-1F** represent urethral myoblast injection. **Figs. 1G-1I** represent urethral myoblast injection using a double staining technique in which both lacZ staining (blue) and fluorescent latex microsphere labeling (fluorescent green) can be visualized. Many regenerative myofibers expressing ß-galactosidase are seen in the urethral and bladder neck wall. There are large, disorganized patterns of myofibers intermingled with fluorescent latex microspheres. Hematoxylin-eosin (H and E) tissue staining was used.

Fig. 2 shows a high magnification (i.e., 100x) of myoblast

injection into the urethral wall as shown in **Fig. 1I**. Transduced myoblasts, myotubes and myofibers expressing ß-galactosidase (blue color, arrows) are seen in the urethral wall near urethral epithelium (arrow heads). H and E tissue staining was used.

Figs. 3A-3F show the results of transducing myoblasts versus synovial cells *in vitro* (Example 9). Synovial cells (**Figs. 3A, 3B**) and myoblasts (**Figs. 3C, 3D**) are from cell lines grown in culture. Both cell lines were infected with an adenovirus vector carrying the LacZ reporter gene using a similar multiplicity of infection (MOI=25). The expression of ß-galactosidase by both cell types was observed using LacZ histochemistry at 2 days (**Figs. 3A, 3C**) and 6 days (**Figs. 3B, 3D**) post-infection. The transduced myoblasts were shown to preserve their ability to differentiate into myotubes expressing ß-galactosidase (**Fig. 3D**). Desmin immunofluorescence of myoblast cultures indicated the presence of multiple, long, pluri-nucleated myotubes where the myoblasts were allowed to differentiate using fusion media (**Fig. 3E**). The amount of ß-galactosidase production by the four different cell cultures at two days post-infection was quantified using the lacZ assay. The immortalized myoblasts produced nearly 5 times more ß-galactosidase than did primary myoblasts, primary synovial cells and immortalized synovial cells. Magnifications A-E: 10X.

Fig. 4 shows interleukin-1 receptor agonist protein (IRAP) production (ng/ml/10^6 cells as measured by ELISA) after either synovial cells (syn) or myoblasts (myo) transduced with adenoviral vector carrying the gene encoding IRAP (ad-IRAP) were used to infect rabbit joint.

Figs. 5A-5D show the results of myoblast-mediated *ex vivo* gene transfer into rabbit meniscus. Myoblasts transduced with an adenovirus vector carrying the gene encoding ß-galactosidase (LacZ) were injected into rabbit meniscus. **Figs. 5A and 5B** show the expression of LacZ in the meniscus following injection and expression of ß-galactosidase.

Fig. 5C shows that LacZ staining is co-localized with fluorescent latex microspheres in the injected area. **Fig. 5D** shows the expression of desmin, a myogenic marker (green fluorescence) showing the presence of muscle cells in the meniscus.

Figs. 6A-6D show the results of myoblast-mediated *ex vivo* gene transfer into rabbit ligament. Myoblasts transduced with an adenovirus vector carrying the gene encoding ß-galactosidase (LacZ) were injected into rabbit ligament. **Figs. 6A and 6B** show the expression of LacZ in the ligament following injection and expression of ß-galactosidase. **Fig. 6C** shows that LacZ staining is co-localized with fluorescent latex microspheres in the injected area. **Fig. 6D** shows the expression of desmin, a myogenic marker (green fluorescence) showing the presence of muscle cells in the ligament.

Figs. 7A-7H depict the characterization of the survival of different populations of muscle-derived cells following transplantation in skeletal muscle. The injection of the muscle-derived cells obtained following preplate #1 was rapidly lost by 48 hours post-injection (**Fig. 7A**): only 17% of the LacZ transgene expression present in the injected myoblasts pre-injection was measured in the injected muscle. The cells isolated at preplate #2 (**Fig. 7E**) led to 55% myoblast loss; preplate #3 (**Fig. 7B**) a 12% loss; and preplate #6 (**Fig. 7F**) a 124% gain in the level of transgene expression present in the cells before transplantation. A 96% loss of the pure population of myoblasts isolated from myofibers was observed at 48 hours post-transplantation (Fiber myoblast, FMb, **Fig. 7C**). Similarly, the immortalized mdx myoblast cell line showed cell loss following transplantation: 93% of the level of transgene expression present in the cell culture post-implantation was seen 2 days post-injection (Mdx cell line, **Fig. 7G**). PP#3 and PP#6 (**Figs. 7D and 7H**) displayed a better cell survival at 2 days post-injection, yet a decrease was observed in the amount of LacZ reporter gene in the injected muscle at 5 days post-injection. However, the

cells which displayed a better survival (PP#3 and PP#6) remained with a higher level of gene transfer at 5 days post-injection. "*" indicates a significant difference (P<0.05) when compared with transduced non-injected myoblasts (0 hour).

Figs. 8A-8D shows the ability of engineered myoblasts expressing anti-inflammatory substance IRAP to circumvent the poor survival of the injected cells. The survival of the myoblasts engineered to express interleukin-1 receptor antagonist protein (IL-1Ra) (**Fig. 8B**) was compared with the non-engineered control cells (**Fig. 8A**). The non-engineered cells were rapidly lost by 48 hours post-injection (Control myoblast). In contrast, the cells engineered to express IL-1Ra significantly reduced the early loss of the injected cells (IL-1Ra expressing myoblast): only 20% of the injected cells were lost at 48 hours post-injection. However, a significant reduction in the amount of β-galactosidase expression was observed at 24 hours post-injection compared with the non-injected myoblasts. A high number of transduced myofibers persisted between day 2 and day 5 following injection (**Figs. 8C, 8D**). The absence of a significant difference for both populations of cells at 0 and 0.5 hours post-injection suggested that the loss of myoblasts was minimal during injection. "*" indicates a significant difference (P<0.05) compared with transduced non-injected myoblasts (0 hour).

Fig. 9 presents the levels of alkaline phosphatase activity (ALP), U/L, after various muscle-derived cell populations (pp1-pp6) are stimulated with osteogenic protein BMP-2, t=30 minutes. The cell types are stromal cells (control) and preplated (pp) cells #1, 2, 3, 5 and 6 as described. The PP#6 corresponds to BMP-2 by producing alkaline phosphatase in a dose dependent manner and at a level similar to that observed with stromal cells (SC)

Fig. 10 presents the percentage of desmin-positive cells following different numbers of doses (100 ng/ml) of BMP-2. It was observed

that stimulation of BMP-2 not only increased the level of alkaline phosphatase expression by the muscle-derived cells, but also decreased the number of desmin positive cells in the population of muscle-derived cells.

Fig.11 shows that injected muscle-derived cells (PP#6) stimulated with BMP-2 and inserted into a theracyte immunoisolation device (described in Example 1) which was implanted subcutaneously are capable of participating in bone formation as seen by von Kossa (mineralization) and hematoxylin/eosin. These results suggest that muscle-derived cells are capable of forming bone.

Fig. 12 shows a schematic representation of the construction of a shuttle plasmid to construct an adeno-associated virus to carry the expression of IGF-1 (muscle injuries), VEGF (bone and cartilage healing), and BMP-2 (bone and cartilage healing). This shuttle plasmid, designated pXX-UF1, is used to construct an adeno-associated virus.

Fig. 13 shows the results of the use of a muscle biopsy for cartilage healing. In the frames shown in Fig. 13, the muscle biopsy is seen to be encasing the cartilage defect at 3 weeks post-injection and muscle and cartilage formation is apparent. The muscle biopsy can therefore be used as a biological scaffold to deliver growth factors, as well as a source of pluripotent muscle-derived cells to improve the healing of cartilage defect. In this figure, "M" represents muscle, while "C" represents cartilage.

Figs. 14A-14C show the results of myoblast-mediated *ex vivo* gene transfer into rabbit cartilage. Myoblasts transduced with an adenovirus vector carrying the gene encoding β-galactosidase (LacZ) were injected into rabbit cartilage. **Figs. 14A and 14B** show the expression of desmin, a myogenic marker (green fluorescence), which reveals the presence of muscle cells in the cartilage. **Fig. 14C** shows the expression of β-galactosidase in the injected cartilage with myoblasts transduced with adenovirus carrying the expression of β-galactosidase.

Figs. 15A-15C show the results of primary muscle-derived cell injection into the lower urinary tract. (Example 4) Myoblasts transduced with an adenovirus vector carrying the gene encoding ß-galactosidase (LacZ) were injected into mouse bladder and urethra. **Fig. 15A** shows the six-month persistence post-injection without damage to the bladder wall. **Fig. 15B** shows the assays for ß-galactosidase in the injected bladder is maintained approximately 66% after 70 days. **Fig. 15C** shows the cross section of a rat urethra. Injection of primary rat muscle-derived cells resulted in a large bulking effect in the urethra wall.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides genetically engineered muscle-derived cells containing at least one heterologous nucleic acid (i.e., exogenous to the muscle cells) encoding a desired gene product, such as a protein, polypeptide, peptide, hormone, metabolite, enzyme, or a trophic factor, including cytokines, in which the gene product(s) is/are expressed in a sustained fashion in the cells, and are delivered therapeutically by the engineered cells to a tissue or organ site to promote healing after injury, or to remedy a localized organ or tissue dysfunction. Tissues and organs suitable for muscle-derived cell-mediated gene delivery according to the present invention include the musculoskeletal system (e.g., joint), bone, and urogenital system (e.g., urethra, bladder, sphincter).

More particularly, the present invention provides such genetically engineered muscle-derived cells, e.g., myoblasts, to improve and expand the treatment of several types of bladder dysfunction including impaired bladder contractility. Also, the present invention provides for the first time the use of skeletal muscle cells for the repair of urinary tract smooth muscle dysfunction.

The present invention further provides a revolutionary new treatment for urinary incontinence caused by urethral and bladder impairment or dysfunction. Men and women afflicted with stress

incontinence are treated by using autologous muscle-derived cell injection (i.e., such as myoblasts, harvested from the patient) to build up and support the urinary sphincter. The present invention relates to muscle-derived cells injected into the bladder wall as a cellular myoplasty technique to improve detrusor contractility and relates to muscle-derived cell- mediated expression of nitric oxide synthase (NOS) as gene therapy for the treatment of lower urinary tract dysfunction.

A number of muscle-derived or myogenic cells are suitable for use in the present invention. Nonlimiting examples of such cells include myoblasts, fibroblasts, adipocytes and muscle-derived stem cells which reside in muscle tissue. Also intended for use in the present invention are skeletal myoblasts from skeletal muscle, particularly for use in the repair of smooth muscle dysfunction in the urinary tract. In the practice of the present invention, muscle-derived cells are capable of delivering genes not only to skeletal and smooth muscle, but also to bone, cartilage, ligaments and meniscus.

In accordance with the present invention, muscle-derived cells, including myoblasts, may be primary cells, cultured cells, or cloned. They may be histocompatible (autologous) or nonhistocompatible (allogeneic) to the recipient, including humans. Such cells are genetically engineered to carry specific genes encoding particular gene products and/or drug products, and can serve as long-term local delivery systems for a variety of treatments, for example, for the treatment of such diseases and pathologies as bladder cancer, transplant rejection, neurogenic bladder conditions, e.g., those secondary to diabetes mellitus, and for the regeneration of muscle and nerve.

Preferred in the present invention are myoblasts and muscle-derived stem cells, and more preferred are autologous myoblasts and muscle-derived stem cells which will not be recognized as foreign to the recipient. In this regard, the myoblasts used for cell-mediated gene transfer

or delivery will desirably be matched vis-à-vis the major histocompatibility locus (MHC or HLA in humans). Such MHC or HLA matched cells may be autologous. Alternatively, the cells may be from a person having the same or a similar MHC or HLA antigen profile. The patient may also be tolerized to the allogeneic MHC antigens. The present invention also encompasses the use of cells lacking MHC Class I and/or II antigens, such as described in U.S. Patent No. 5,538,722.

Myoblasts, the mononucleated muscle cells, are uniquely different from other cells in the body in a number of ways: l) myoblasts naturally differentiate to form muscle tubules capable of muscle contraction, 2) when myoblasts fuse to form myotubes, these cells become post mitotic (stop dividing) with maturation, thus allowing control of the number and amount of myoblasts per injection, and 3) as myotubes, the cells express large amounts of protein which is produced in the cells due to multinucleation.

In accordance with the present invention, muscle-derived cells, including myoblasts, may be genetically engineered by a variety of molecular techniques and methods known to those having skill in the art, for example, transfection, infection, or transduction. Transduction as used herein refers to cells which have been genetically engineered to contain a foreign or heterologous gene via the introduction of a viral vector into the cells. Muscle-derived cells, including myoblasts, can be transduced by different viral vectors and thus can serve as gene delivery vehicles to transfer expressed proteins into muscle.

Although viral vectors are preferred, those having skill in the art will appreciate that the genetic engineering of cells to contain nucleic acid sequences encoding desired proteins or polypeptides, cytokines, and the like, may be carried out by methods known in the art, for example, as described in U.S. Patent No. 5,538,722, including fusion, transfection, lipofection mediated by the use of liposomes, electroporation, precipitation

with DEAE-Dextran or calcium phosphate, particle bombardment (biolistics) with nucleic acid-coated particles (e.g., gold particles), microinjection, and the like.

The present invention also relates to vehicles or vector constructs for introducing heterologous (i.e., foreign) nucleic acid (DNA or RNA), or a segment of nucleic acid that encodes a functional bioactive product, into muscle-derived cells, in which the vectors comprise a nucleic acid sequence read in the correct phase for expression. Such vectors or vehicles will, of course, possess a promoter sequence, advantageously placed upstream of the sequence to be expressed. The vectors may also contain, optionally, one or more expressible marker genes for expression as an indication of successful transfection and expression of the nucleic acid sequences contained in the vector. To insure expression, the vectors contain a promoter sequence for binding of the appropriate cellular RNA polymerase, which will depend on the cell into which the vector has been introduced. For example, the promoter for expression in muscle-derived cells, such as myoblasts, is a promoter sequence to which the cellular RNA polymerases will bind.

Illustrative examples of vehicles or vector constructs for transfection or infection of muscle-derived cells include replication-defective viral vectors, DNA virus or RNA virus (retrovirus) vectors, such as adenovirus, herpes simplex virus and adeno-associated viral vectors. Adeno-associated virus vectors are single stranded and allow the efficient delivery of multiple copies of nucleic acid to the cell's nucleus. Preferred are adenovirus vectors. The vectors will normally be substantially free of any prokaryotic DNA and may comprise a number of different functional nucleic acid sequences. An example of such functional sequences may be a DNA region comprising transcriptional and translational initiation and termination regulatory sequences, including promoters (e.g., strong promoters, inducible promoters, and the like) and enhancers which are

active in muscle cells. Also included as part of the functional sequences is an open reading frame encoding a protein of interest, and may also comprise flanking sequences for site-directed integration. As a particular example, in some situations, the 5'-flanking sequence will allow homologous recombination, thus changing the nature of the transcriptional initiation region, so as to provide for inducible or noninducible transcription to increase or decrease the level of transcription, as an example.

In general, the nucleic acid desired to be expressed by the muscle-derived cell is that of a structural gene, or a functional fragment, segment or portion of the gene, that is heterologous to the muscle-derived cell and encodes a desired protein or polypeptide product, for example. The encoded and expressed product may be intracellular, i.e., retained in the cytoplasm, nucleus, or an organelle of a cell, or may be secreted by the cell. For secretion, the natural signal sequence present in the structural gene may be retained, or a signal sequence that is not naturally present in the structural gene may be used. When the polypeptide or peptide is a fragment of a protein that is larger, a signal sequence may be provided so that, upon secretion and processing at the processing site, the desired protein will have the natural sequence. More specific examples of genes of interest for use in accordance with the present invention include the genes encoding nitric oxide synthase; trophic factors, including growth factors and cytokines, such as basic and acidic fibroblast growth factors (bFGF and aFGF), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin, insulin-like growth factor (IGF), transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-ß), platelet derived growth factor (PDGF) and the like; hormones; metabolic products, generally of low molecular weight; diffusable products; serum proteins; osteogenic proteins, e.g., BMP-2.

As mentioned above, a marker may be present for selection of cells containing the vector construct. The marker may be an inducible or

non-inducible gene and will generally allow for positive selection under induction, or without induction, respectively. Examples of marker genes include neomycin, dyhydrofolate reductase, LacZ, and the like.

The vector employed will generally also include an origin of replication and other genes that are necessary for replication in the host cells, as routinely employed by those having skill in the art. As an example, the replication system comprising the origin of replication and any proteins associated with replication encoded by a particular virus may be included as part of the construct. As a caveat, the replication system must be selected so that the genes encoding products necessary for replication do not ultimately transform the muscle-derived cells. Such replication systems are represented by replication-defective adenovirus constructed as described by G. Acsadi et al., 1994, *Human Mol. Genetics*, 3(4):579-584, and by Epstein-Barr virus. Examples of replication defective vectors, particularly, retroviral vectors that are replication defective, are BAG, described by Price et al., 1987, *Proc. Natl. Acad. Sci.*, 84:156; and Sanes et al., 1986, *EMBO J.*, 5:3133. It will be understood that the final gene construct may contain one or more genes of interest, for example, a gene encoding a bioactive metabolic molecule, e.g., NOS, iNOS, or NO, and a gene encoding a cytokine, e.g., bFGF, along with the sequences allowing for the proper expression and production of the gene products by the engineered cells. In addition, cDNA, synthetically produced DNA or chromosomal DNA may be employed utilizing methods and protocols known and practiced by those having skill in the art.

If desired, infectious replication-defective viral vectors may be used to genetically engineer the cells prior to *in vivo* injection of the cells. In this regard, the vectors may be introduced into retroviral producer cells for amphotrophic packaging. The natural expansion of muscle-derived cells, such as myoblasts, into adjacent regions obviates a large number of injections into the muscle fibers at the site(s) of interest.

In one embodiment of the present invention muscle-derived cells are transduced with nucleic acid encoding a particular gene product, e.g., a gene encoding inducible nitric oxide synthase (iNOS). The transduced muscle-derived cells, which contain, express and produce the iNOS product, are used in cell-mediated transplantation or gene therapy techniques for the treatment of genitourinary tract dysfunction. Examples of lower urinary tract dysfunction include, but are not limited to, erectile dysfunction of the penis, pyronies disease of the penis; dysfunctions of the urethra, such as stress urinary incontinence, bladder outlet obstruction, urethritis, dysfunction voider; bladder dysfunctions, such as impaired bladder contractility, neurogenic bladder, cystitis and bladder inflammatory disease; and dysfunction of the female sexual and reproductive organs, such as vagina, cervix, uterus, fallopian tubes and ovaries.

The same or different muscle-derived cells may also be co-transduced with heterologous nucleic acid encoding trophic factors whose expression in and production by the muscle-derived cells aid in effecting and/or enhancing the therapeutic uses of the transduced muscle-derived cells in cell-mediated gene therapy. Trophic factors such as cytokines are preferably used. More specifically, useful cytokines include those presented hereinabove, among which are basic fibroblast growth factor (bFGF), nerve growth factor (NGF) and interleukins, such as IL-1 and IL-6.

Muscle-derived cells engineered to contain nucleic acid encoding one or more trophic factors can be administered as a treatment at the same time as muscle-derived cells containing nucleic acid encoding a therapeutic protein or a bioactive molecule, such as a protein, polypeptide, peptide, hormone, metabolite, drug, enzyme, and the like. Alternatively, muscle-derived cells engineered to express trophic factors may be administered at a later or earlier time, depending on the type of treatment desired.

In general, an injection of genetically engineered muscle-

derived cells, including myoblasts and muscle-derived stem cells, into a given tissue or site of injury comprises a therapeutically effective amount of cells in solution or suspension, preferably, about 10^5 to 10^6 cells per cm^3 of tissue to be treated, in a physiologically acceptable medium, such as saline or phosphate buffered saline, and the like.

In a preferred aspect, the present invention provides *ex vivo* gene delivery to cells and tissues of a recipient mammalian host, including humans, through the use of muscle-derived cells, e.g., myoblasts, that have been virally transduced using an adenoviral vector engineered to contain a heterologous gene encoding a desired gene product. Such an *ex vivo* approach provides the advantage of efficient viral gene transfer which, in cases of treatment of muscle-related dysfunction and defects as described herein, is superior to direct gene transfer approaches. The *ex vivo* procedure involves the establishment of a primary muscle-derived cell culture from isolated cells of muscle tissue. The muscle biopsy that will serve as the source of muscle-derived cells can be obtained from the injury site or from another area that may be more easily obtainable from the clinical surgeon.

The muscle-derived cells are first infected with engineered viral vectors containing at least one heterologous gene encoding a desired gene product, and then are injected into the same host. In the case of myoblasts as an example, the injected, transduced, isogenic muscle-derived cells then fuse to form myotubes at and near the site of injection. The desired gene product is expressed by the injected cells which thus introduce the gene product into the injected tissue, e.g., muscle. The introduced gene products can promote and enhance muscle regeneration and muscle strength *in vivo* to ameliorate muscle healing following injuries.

In a particular embodiment of the present invention, muscle-derived cell injection, preferably autologous myoblast injection, into the urethral wall is employed as a treatment for stress urinary incontinence to

enhance, improve, and/or repair the urinary sphincter. Muscle-derived cells, preferably myoblasts, carrying one or more transduced or transfected heterologous nucleic acids encoding a bioactive molecule and/or a trophic factor, are injected into the urethral wall and survive and differentiate into myofibers to improve sphincter function. The feasibility and survival of myoblast injection into the urethral wall is demonstrated in Example 2. In accordance with this embodiment, autologous muscle-derived cell injections (i.e., muscle-derived cells harvested from and cultured for a specific stress incontinence patient) can be used as a nonallergenic agent to bulk up the urethral wall, thereby enhancing coaptation and improving the urinary sphincter muscle.

In another embodiment of the present invention, muscle-derived cells are injected into the bladder wall to improve detrusor contractility. Muscle-derived cells, such as myoblasts and muscle-derived stem cells, injected into the bladder wall are capable of surviving and differentiating into myofibers that can augment detrusor contractility as demonstrated in Example 3. In addition, muscle-derived cells which have been genetically engineered to carry a foreign gene express the foreign gene product after injection into the bladder wall.

In accordance with the present invention, autologous muscle-derived cells administered directly into the bladder and urethra exhibit long-term survival. The use of cell-mediated gene therapy involving genetically engineered muscle-derived cells is advantageous over the use of other forms of gene therapy, i.e., gene therapies involving the direct administration of virus or plasmid vectors, for example. As an example, the type of muscle-derived cell may contribute to the survival of the injected cells post-transplantation. In this regard, autologous primary myoblasts can be harvested and cultured myoblasts can be stored and used in sufficient quantities for repeated urethral and bladder injections. Autologous myoblast injection results in safe and nonimmunogenic long-term survival of

myofibers in the lower urinary tract. (see Example 4 and Figs. 15A-15C).

In a particular embodiment, muscle-derived cell mediated gene therapy of the present invention further involves muscle-derived cells, e.g., myoblasts, transduced with an adenovirus vector carrying the bFGF gene, thereby allowing expression of bFGF by the transduced muscle-derived cells in a given tissue. bFGF engineered muscle-derived cells are injected into the urethra to treat stress incontinence and also into the bladder wall to improve detrusor contractility. In accordance with the present invention, injection of bFGF engineered muscle-derived cells allows improvement in survival and function versus non-engineered muscle-derived cells. Following short-term experiments, long-term experiments were conducted using autologous primary myoblast-bFGF injection into the bladder and urethra at 4, 14, and 30 days, as described in Example 6. Cell mediated gene therapy using transduced myoblasts which secreted the trophic factor bFGF provided further improved success in overcoming dysfunctions in the urethra and bladder compared with non-bFGF-secreting myoblasts.

In another preferred embodiment, adenoviral vectors carrying the inducible nitric oxide synthase (iNOS) gene are introduced into muscle-derived cells and the resulting transduced cells are used in cell-mediated gene therapy. When such transduced muscle-derived cells are administered locally to the urethra and bladder, dramatic functional modifications are demonstrated, e.g., decrease in bladder inflammation and improvement in urethral relaxation (see Example 7). In this aspect of the present invention, iNOS engineered muscle-derived cell-mediated gene therapy in the bladder provided a diminution of bladder inflammation. In addition, iNOS engineered myoblast injection into the urethra was demonstrated to be useful to decrease urethral outlet obstruction. According to the present invention, myoblast mediated iNOS gene therapy resulted in increased local NO production in injected tissue. iNOS gene

therapy in the bladder also decreased the cyclophosphamide (CYP)-induced bladder inflammatory response. iNOS gene therapy into the urethra further induced sustained urethral smooth muscle relaxation as described in Example 7.

In another embodiment of the present invention, the use of muscle-derived cell-mediated gene transfer to the musculoskeletal system, such as the joint, offers numerous advantages. A muscle biopsy for the isolation of muscle-derived cells, e.g., myoblasts, for use as gene delivery vehicles in accordance with the present invention is much easier and less invasive than, for example, surgical synovial capsule biopsy for the isolation of synovial cells. In a muscle biopsy, a small area of muscle tissue generally contains enough myogenic cells to produce quickly millions of muscle-derived cells in culture. For myoblasts, once the cells are isolated and grown in culture, it is easy to distinguish pure myoblasts from other cell types, since myoblasts fuse to form elongated myotubes *in vitro*. In addition, desmin, a myogenic specific marker protein, can be used to determine the myogenicity index of the cell culture without the requirement of differentiation. In contrast, because synovial cells are more difficult to distinguish from other cell types, obtaining a pure synovial cell culture is problematic for the treatment of muscle disease or dysfunction.

In addition, before fusion, myoblasts are over five times more efficiently transduced than synovial cells using the same number of adenoviral particles per cell *in vitro*. The differentiation of transduced myoblasts into myotubes increases the level of gene expression in the differentiated myotubes and myofibers. One of the major advantages of myoblasts over other cell types, such as synovial cells, is the myoblast's ability to fuse and become a differentiated post-mitotic cell, which can persistently express a high level of an exogenous gene product. In fact, myoblast mediated gene expression has persisted for at least 35 days in the joint. This allows for the persistent and efficient expression and

production of any proteins of interest in the joint.

In addition, myoblasts transduced with adenoviral vectors carrying the gene encoding interleukin-1 receptor agonist protein (IRAP) produced nine times more IRAP as measured by ELISA than infection by similarly transduced synovial cells. The results indicate that myoblasts are introducing more protein following gene transfer into the knee joint synovial lining adjacent to the patella. (Fig. 4).

Moreover, myoblasts have been demonstrated to survive in newborn and adult articular joints (knee) of animal models. Such myoblasts have adhered to most of the structures of a newborn knee, including patellar ligaments, cruciate ligaments, meniscus, synovium and joint capsule. By contrast, synovial cells have survived in the knee by adhering only to the synovial lining.

In addition, injections of muscle-derived cells directly into the intra-articular structures, including ligament and meniscus, results in a higher gene transfer than that observed using injection into the joint fluid. By the practice of the present invention, it has been shown that myoblasts engineered with an adenovirus carrying the expression of the β-galactosidase reporter gene are capable of delivering a higher efficiency and a long term persistence of the reporter gene when compared to the use of ligament fibroblasts and direct adenoviral injection (Figs. 6A-6D) Accordingly, adenovirally transduced myoblasts were able to deliver genes and express gene products in the ligament and meniscus of rabbit at 1 week post injection (Figs. 5A-5D, meniscus and Fig. 6A-6D, ligament), thereby indicating that that the use of this technology can help to improve ligament healing following injuries. Thus, as described herein, injection directly into the intra-articular structures or into joint fluid can be used to deliver genes into the joint.

Stable and persistent gene delivery via muscle-derived cells in the joints has numerous clinical applications. Genetically engineered

muscle-derived cells, such as myoblasts and muscle-derived stem cells, can deliver proteins with an anti-arthritic function, such as interleukin-1 receptor agonist protein (IL-1Ra) or soluble receptors for tumor necrosis factor-α (TNF-α) into an inflamed knee joint (Example 9). This technology can supplant surgical intervention to aid in the healing of different types of damaged tissues (e.g., ligament, meniscus, cartilage) which have poor intrinsic healing capacities in the joint. Also, growth factors can be delivered to the site and persist there in the practice of the present invention to ameliorate tissue pathologies in the joint. The high level of gene expression and gene product production by the muscle-cell mediated gene transfer technology provides advantages to the art that were previously unable to be attained using current methodologies.

In accordance with the present invention, muscle-derived stem cells can be isolated from skeletal muscle. When appropriately stimulated with bone morphogenetic protein-2 (BMP-2), these cells were capable of expressing alkaline phosphatase in a dose dependent manner and, more importantly, of actively participating in bone formation *in vivo*. These cells have the ability to differentiate into other lineages. Accordingly, these cells may be used not only to deliver growth factors and cytokines for the musculoskeletal system, but also to act as an endogenous source of responding cells that may actively participate in the healing process of the musculoskeletal system.

In another embodiment of the present invention, muscle-derived cell-mediated gene transfer is employed to treat bone defects (Example 10). Because most bone defects are surrounded by muscle, the injected muscle-derived cells, such as myoblasts, have a natural myogenic milieu to fuse onto. Muscle biopsies to isolate muscle-derived cells suitable for use according to the invention are less invasive procedures than are bone marrow biopsies to isolate stromal cells, for example. In fact, most muscle biopsies can be done in an office setting.

In addition, a large percentage of the cells isolated from a skeletal muscle biopsy are myoblasts, while only 1 to 2% of bone marrow cells have osteogenic capacity. Myoblasts in cell culture can be further purified using an established technology (T.A. Rando and H.M. Blau, 1994, *J. Cell. Biol.*, 125:1275-1287) and muscle cells are relatively easy to cultivate *in vitro*; millions of cells can be grown in a few days.

Another advantage of muscle-derived cell mediated gene therapy for the treatment of bone defects is that muscle cells can transform into osteoblasts when stimulated by osteogenic proteins, thereby affording them osteogenic potential after stimulation. For example, transduced myoblasts were capable of fusing into myofibers in the bone defect and in the muscles surrounding the defect while expressing a marker gene (Example 10). Thus, in accordance with the present invention, engineering the skeletal muscle cells surrounding the bone defect to express or overexpress osteogenic proteins, e.g., BMP-2, allows the release of these proteins to improve bone healing at the adjacent bone defect. More importantly, the transplantation of BMP-2 engineered muscle-derived cells, which can fuse to form myotubes and myofibers producing the osteogenic protein in the bone defect, can further enhance bone healing. In fact, muscle-derived cell mediated gene transfer of osteogenic proteins has the capability of improving both osteoinductive and osteoconductive aspects of bone healing.

The cyto-architecture of the fused myofibers may not only serve as a reservoir of secreting osteogenic proteins, but also as a matrix for bone formation. It is likely that muscle, due to its high vascularity, may improve the revascularization of the bone defect and, therefore, aid in the improvement of bone healing. In accordance with this embodiment, the present invention provides a viable and efficient approach to improving the healing of both segmental bone defects, bone fractures and non-unions, and provides cells which can participate in desired bone formation.